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Title: Biofilm Formation by *Salmonella Spp.* on Cantaloupe Melons

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BIOFILM FORMATION BY *SALMONELLA* SPP. ON CANTALOUPE MELONS*

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ABSTRACT

The ability of two strains of Salmonella *to form biofilms on whole cantaloupe melons was investigated. Ten microliters of bacterial suspensions was spot-inoculated onto cantaloupe melon rinds in pre-marked areas, and the cantaloupe melons were held at either 10 or 20C. Biofilm formation was monitored using scanning electron microscopy on excised portions of the cantaloupe melon rind at 2, 24, 48, 72 and 144 h postinoculation. Micrographs indicated that biofilm formation occurred rapidly following introduction of cells (2 h at 20C) onto the cantaloupe melon rind. A fibrillar material was visible after just 2 h at 20C, and cells were embedded in extracellular polymeric material after 24 h at either temperature. These results indicate that a human pathogen is capable of forming a biofilm on plant tissue and that biofilm formation may be responsible for the increased recalcitrance of bacteria to aqueous sanitizers.*

INTRODUCTION

In recent years, *Salmonella* spp. have been implicated in outbreaks of foodborne illness linked to the consumption of fresh fruits, most notably, cantaloupe melons. At least six multistate outbreaks of salmonellosis have been traced to the consumption of cantaloupe since 1990, when an outbreak of

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* Mention of brand or firm name does not constitute an endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

Salmonella enterica sv. Chester caused 245 illnesses in 30 states (Ries *et al.* 1990). In 1991, an outbreak of *S. enterica* sv. Poona involving 400 infections in 23 states led to a U.S. Food and Drug Administration (FDA) microbiological survey of imported cantaloupe (CDC 1991). The results of the survey indicated contamination of approximately 1% of the rind of cantaloupe melons with various *Salmonella* serovars. A 1997 outbreak of *S. enterica* sv. Saphra (Mohle-Boetani *et al.* 1999) was attributed to the consumption of cantaloupes imported from Mexico. A second FDA survey conducted in response to this outbreak found that approximately 5% of imported cantaloupes were positive for *Salmonella* (FDA 2001). Three successive outbreaks of *S. enterica* sv. Poona (April–June, 2000; April–May, 2001 and March–May, 2002) that resulted in 154 cases were attributed to the consumption of cantaloupe melons imported from Mexico (CDC 2002). Thus, on October 28, 2002, the FDA issued an import alert detaining all cantaloupes from Mexico offered for entry at U.S. ports (FDA 2002).

Previous research in our laboratory has documented the inability of a variety of sanitizers and other treatments to completely remove and/or inactivate *Salmonella* inoculated onto cantaloupes (Ukuku and Sapers 2001; Sapers and Sites 2003; Annous *et al.* 2004, 2005). In addition, the efficacy of rinsing with chlorine and hydrogen peroxide decreased significantly when the organism was allowed to reside on the cantaloupe melon surface for more than 24 h (Ukuku and Sapers 2001). Ukuku and Sapers (2001) speculated that increased contact time allowed for strong microbial attachment to the cantaloupe melon surface and the formation of a bacterial biofilm prior to sanitation. Experiments conducted to assess the relative strength of attachment (S_R) of *Salmonella* on cantaloupe rinds demonstrated an increasing strength of attachment from days 0–7 during storage (Ukuku and Fett 2002).

A *bacterial biofilm* is generally defined as "an assemblage of microorganisms adherent to each other and/or to a surface and embedded in a matrix of exopolymers" (Costerton *et al.* 1999). Considerable evidence proves that bacteria readily form biofilms on the tissues of numerous plant species (Morris *et al.* 1997; Fett and Cooke 2003; Annous *et al.* 2004). While large numbers of single cells do occur on plants, biofilms may constitute between 30 and 80% of the total population on plant surfaces (Lindow and Brandl 2003). The formation of biofilms by bacteria on plant surfaces likely improves the ability of these organisms to colonize and survive the harsh environment of the phyllosphere. Biofilm-associated bacteria embedded in a matrix of extracellular polysaccharides (EPS) might be more difficult to remove from contaminated surfaces than their solitary counterparts (Fett and Cooke 2003; Annous *et al.* 2004). In addition, the production of EPS likely shields bacterial cells within biofilms from desiccation and aids in the resistance to antimicrobial compounds.

Biofilm formation by *Salmonella* has only recently been investigated Zogaj *et al.* 2001; Solano *et al.* 2002). The formation of biofilms by cells of *Salmonella* has been documented on various inert surfaces (i.e., glass, cement and rubber) (Sommers *et al.* 1994; Joseph *et al.* 2001). Two components of the extracellular matrix produced by *Salmonella* have recently been elucidated: thin aggregative fimbriae and cellulose. The production of cellulose has been linked to increased resistance of *Salmonella* to chlorine (Solano *et al.* 2002).

The formation of aggregates by *Salmonella* spp. on cilantro and *Arabidopsis* plants has recently been demonstrated (Brandl and Mandrell 2002; Cooley *et al.* 2003). The objective of our research was to demonstrate biofilm formation by *Salmonella* spp., associated with cantaloupe outbreaks, on the rind of cantaloupes.

MATERIALS AND METHODS

Bacteria

Two clinical isolates associated with cantaloupe outbreaks, *S. enterica* sv. Poona RM 2350 (California Department of Health 00A3563) and *S. enterica* sv. Michigan, were obtained from Dr. William Fett (U.S. Department of Agriculture (USDA), Agricultural Research Service, Eastern Regional Research Center, Wyndmoor, PA). The stocks were stored in tryptic soy broth (TSB) (BBL/Difco, Sparks, MD) containing 30% glycerol, at –80C. The working cultures were maintained on tryptic soy agar (TSA) (BBL/Difco, Sparks, MD) slants at 4C.

Inoculum Preparation and Inoculation Methods

A loopful of culture from a TSA slant was transferred into 10 mL of TSB and allowed to grow for 8 h at 37C. This culture (10 μL) was then used to inoculate 100 mL of fresh TSB medium. The culture was allowed to grow at 37C for 18 h, centrifuged (6740 × g, 20 min), washed with 100-mL sterile deionized water, recentrifuged and the final cell pellet was suspended in 100-mL sterile deionized water to give a final cell concentration of *c.* 9 $\log_{10}$ cfu/mL (enumerated on TSA). Nine cantaloupes were used with each strain. Each of the nine cantaloupes was spot-inoculated by applying 10 μL of the inoculum described above to the center of each of four 10-mm diameter circles marked on the rind of cantaloupe melon with a nontoxic, permanent marking pen (Sharpie, Series No. 37000; Sanford, Bellwood, IL). The inoculated cantaloupe melons were allowed to dry in a biosafety cabinet for 2 h at room temperature (RT = 19 ± 1C). After drying, four cantaloupe melons

inoculated per strain were placed into paper-lined tubs (Rubbermaid, Wooster, OH) covered with aluminum foil, and the tubs were placed onto the laboratory bench at RT. Four other cantaloupes were placed into a second tub, covered as above and placed into a 10C incubator. The remaining inoculated cantaloupe melon per strain was sampled immediately after drying for 2 h. Noninoculated (negative) controls were included for each sampling time.

Sampling Method and Scanning Electron Microscopy

The cantaloupe melons were sampled at 2, 24, 48, 72 and 144 h following inoculation. The cantaloupe melon rinds onto which the inoculum had been deposited were removed with a sterile 12.3-mm cork borer, and the adhering flesh tissue was removed with a knife. The rind plugs were placed into petri dishes lined with sterile filter paper. Duplicate samples were initially fixed in glutaraldehyde vapor to avoid washing off dried layers of inoculum on the rind surface, followed by immersion fixation in 2.5% glutaraldehyde–0.1 M imidazole buffer solution (pH = 7.0) for 2 h and stored in sealed bottles at RT until further processing. The fixed samples were then washed in the buffer, dehydrated in a graded series of ethanol (50, 80%, then absolute) and critical-point-dried from liquid carbon dioxide. The plugs were mounted onto specimen stubs using Duco cement (Devcon, Riviera Beach, FL) and coated with a thin layer of gold by direct current sputtering. Digital images were collected in the secondary electron-imaging mode of a scanning electron microscope (SEM) Model Quanta 200 (FEI, Hillsboro, OR).

RESULTS AND DISCUSSION

Native microbial populations of *c.* 4.7 log cfu/cm^2 (data not shown) on the surface of the rind of cantaloupe melons were difficult to visualize by SEM because of the uneven morphology of the cantaloupe netting (Fig. 1) and the low initial levels of native microflora present on cantaloupe melons (Fig. 2). This is in contrast with native biofilm detected on smooth plant surfaces such as alfalfa and other types of sprouts, endive and bean (Morris *et al.* 1997; Fett and Cooke 2003). Because of the lack of visible microflora on negative control samples (Fig. 2), biofilm formation observed within the marked areas where the inoculum had been applied was considered to be composed of *Salmonella* cells.

We previously reported an increase (2–3 log cfu/cm^2) in the population of *S. enterica* sv. Poona RM 2350 on the rind of inoculated cantaloupe melons during storage at RT for up to 72 h (Annous *et al.* 2004, 2005), indicating that the rind of cantaloupe melon is able to support the growth of this microorgan-

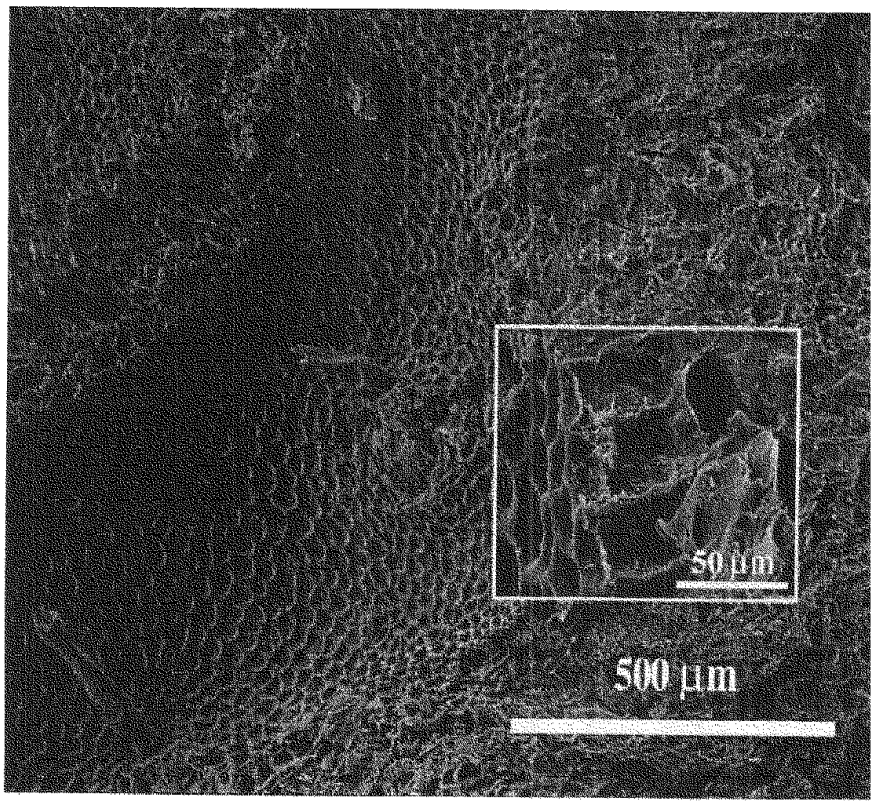

FIG. 1. SCANNING ELECTRON MICROGRAPH (×100) OF A CANTALOUPE RIND SHOWING AN EXTENSIVE, DEEPLY FISSURED REGION OF THE SURFACE (NETTING)
The insert image is an enlargement of a section of the netting (×1000) showing an extensive compartmentalization of the tissues.

ism. The growth of *Salmonella* cells on the rind might contribute to the biofilm formation and the subsequent increase in resistance to sanitizers. The inoculated cantaloupe melons that were stored at 4C for up to 72 h showed a reduction in *S. enterica* sv. Poona populations in excess of 1 log cfu/cm^2 (Annous *et al.* 2004). These results indicated that refrigeration can suppress the growth of this microorganism and could decrease the microbial safety risk associated with this commodity. This prompted us to investigate the effect of storage temperature on biofilm formation by *Salmonella* spp. on the rind of cantaloupe melons. A storage temperature of RT or 10C was chosen to simulate temperature abuse during storage.

Both *S. enterica* sv. Poona RM 2350 and *S. enterica* sv. Michigan were found to produce large amounts of biofilm following their introduction onto the cantaloupe melon rind (Figs. 3–5). A fibrillar material was clearly evident

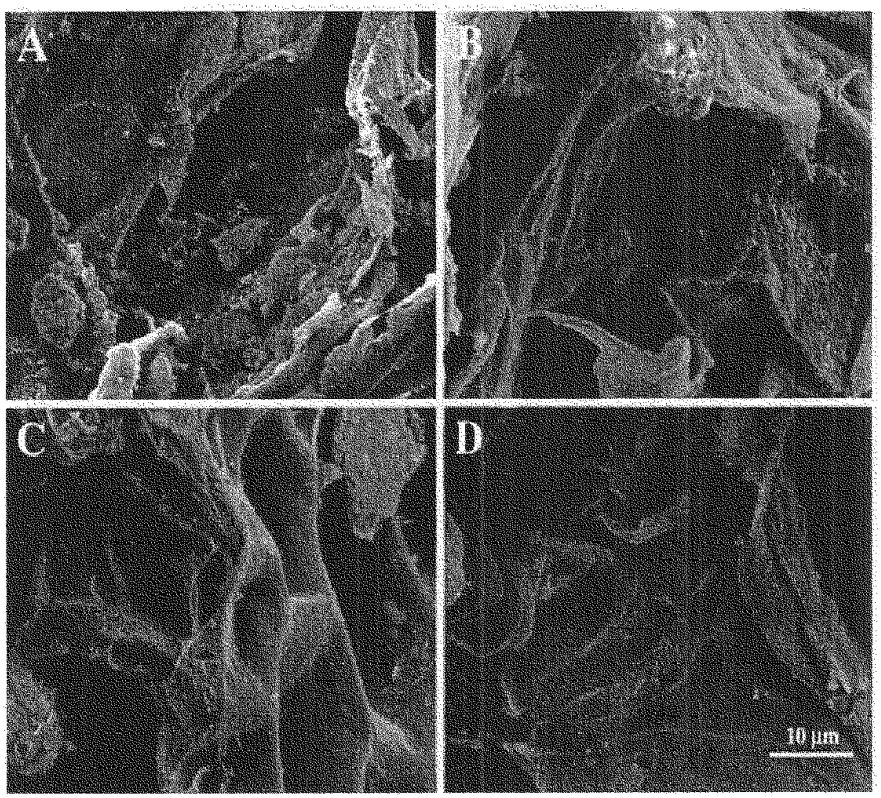

FIG. 2. SCANNING ELECTRON MICROGRAPH (×2500) SHOWING THE NETTING OF CONTROL (UNINOCULATED) CANTALOUPE FOLLOWING STORAGE AT 10C FOR (A) 48 OR (B) 72 H, AND 20C FOR (C) 48 OR (D) 72 H
Little native microflora is visible on the surface of the rind.

within 2 h of inoculation following drying at RT (Fig. 3A). Biofilm formation occurred rapidly, with cells embedded in polymeric material after just 24 h of residence time on the rind tissue, regardless of temperature (Figs. 3B, 4B and 5A,C). Additionally, cells of *Salmonella* were arranged into latticelike matrices on the surface of the netting following 24 h of incubation (Figs. 3B and 4B). These lattices soon became covered with a sheetlike material, strengthening further the attachment of the bacterial cells to the netting (Figs. 3C,D; 4C,D and 5C,D). This sheetlike material likely provides protective covering for the *Salmonella* cells and may be involved in their resistance against aqueous sanitizers.

The surface of the cantaloupe melon, with its meshwork of lenticellar netting (Fig. 1), provides a large number of attachment sites for *Salmonella* that aid in avoiding contact with aqueous sanitizers (Annous *et al.* 2004).

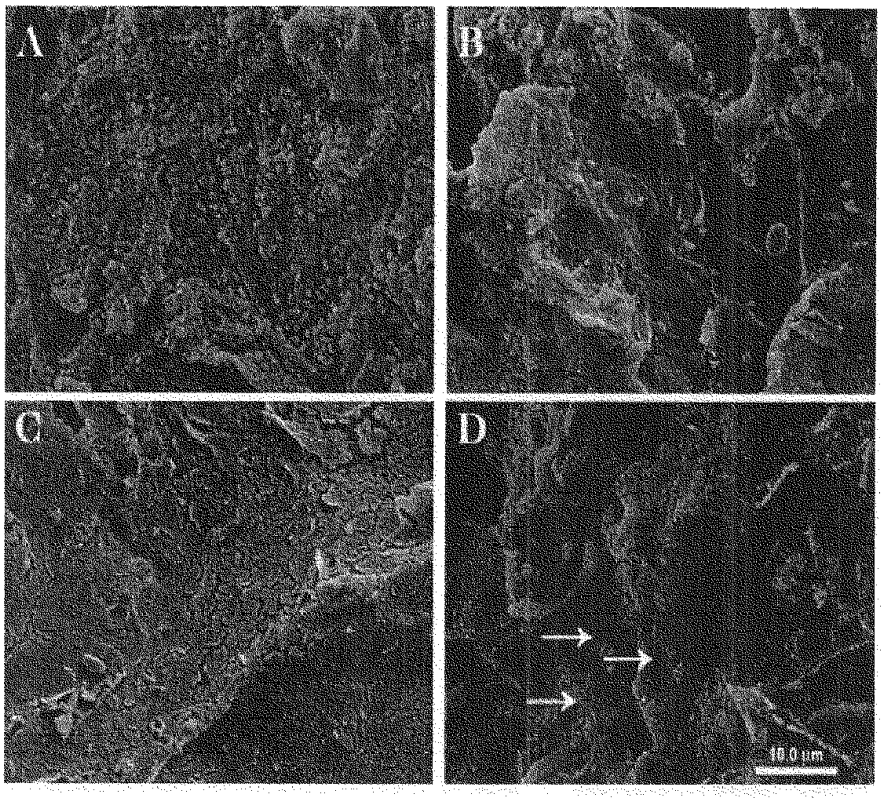

FIG. 3. SCANNING ELECTRON MICROGRAPH (×2500) SHOWING ATTACHMENT AND BIOFILM FORMATION BY *SALMONELLA ENTERICA* SV. MICHIGAN CELLS INSIDE THE NETTING OF INOCULATED CANTALOUPES FOLLOWING STORAGE AT 20C
(A) For 2 h. Note that the fibrillar material is already visible. (B) For 24 h. The cells are visibly arranged within the cracks on the netting. (C) For 48 h. Note the extracellular matrix encapsulating cells. (D) For 72 h. Note the filamentous fungi interspersed with bacterial cells (arrows).

Depositions of waxy cutin render guard cells nonfunctional in maturing fruit, leaving lenticels open (Webster and Craig 1976). As the fruits mature, shallow cracks in the cuticle become large, contiguous fissures (Webster and Craig 1976; Lester 1988). The hydrophobic cuticle, present on the surfaces of a large number of fruits and vegetables, normally prevents the infiltration of phytopathogens (Richards and Beuchat 2004). Disruptions in this cuticle have been shown to enhance the ability of foodborne pathogens to attach to plant tissue (Han *et al.* 2000; Kenney *et al.* 2001). Clearly, these fissures present within the cuticle on the cantaloupe melon surface lead to greater infiltration and attachment of *Salmonella* cells.

Salmonella cells that come in contact with the surface of cantaloupe melons in the field could easily become embedded within the fissures in the

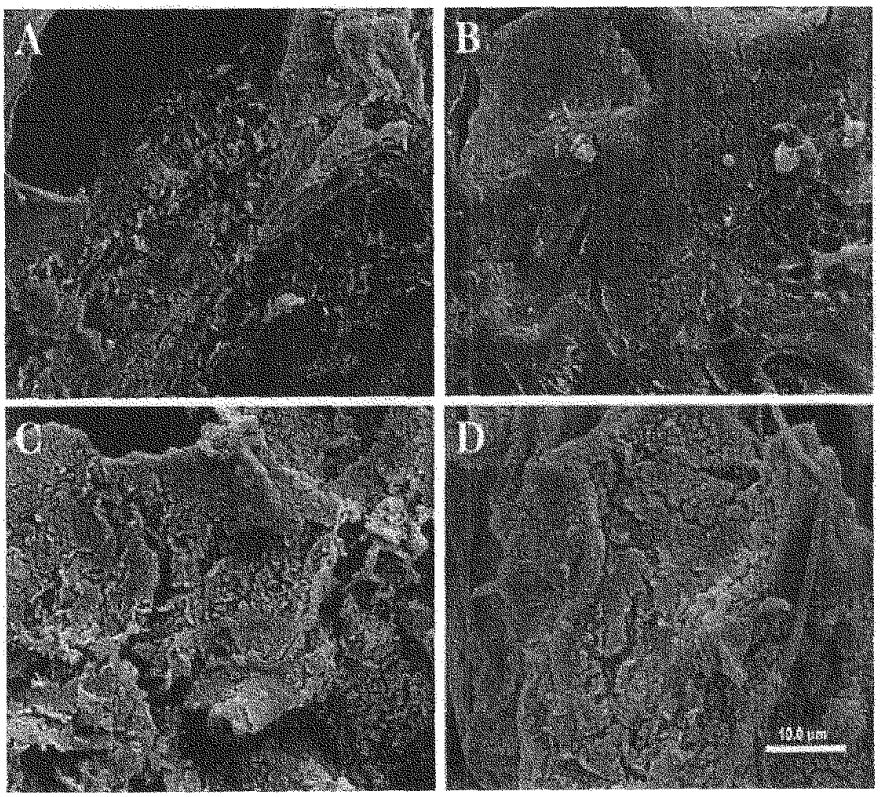

FIG. 4. SCANNING ELECTRON MICROGRAPH (×2500) SHOWING ATTACHMENT AND BIOFILM FORMATION BY *SALMONELLA ENTERICA* SV. POONA RM 2350 CELLS INSIDE THE NETTING OF INOCULATED CANTALOUPES FOLLOWING STORAGE AT 20C
(A) For 2 h. (B) For 24 h. Note large aggregates of cells held together by an extracellular matrix. (C) For 48 h. The cells are once again arranged within the cracks on the netting. (D) For 72 h.

cuticle, protected from environmental stress and can survive through harvest and transport periods, as evidenced by the large number of positive samples found during surveys of cantaloupe (FDA 2001, 2003). Surviving *Salmonella* could then be transferred from the surface of the cantaloupe melon into the internal tissues during cutting prior to consumption (Ukuku and Sapers 2001). In addition, the marketing of freshly cut fruits at improper storage temperatures could lead to the rapid growth of surviving cells (Golden *et al.* 1993; Annous *et al.* 2004).

Biofilm formation by foodborne pathogens on plant tissue has only recently been examined. Brandl and Mandrell (2002) demonstrated the formation of large heterogeneous aggregates by *S. enterica* sv. Thompson on cilantro leaves. The aggregate formation was dependent on storage tempera-

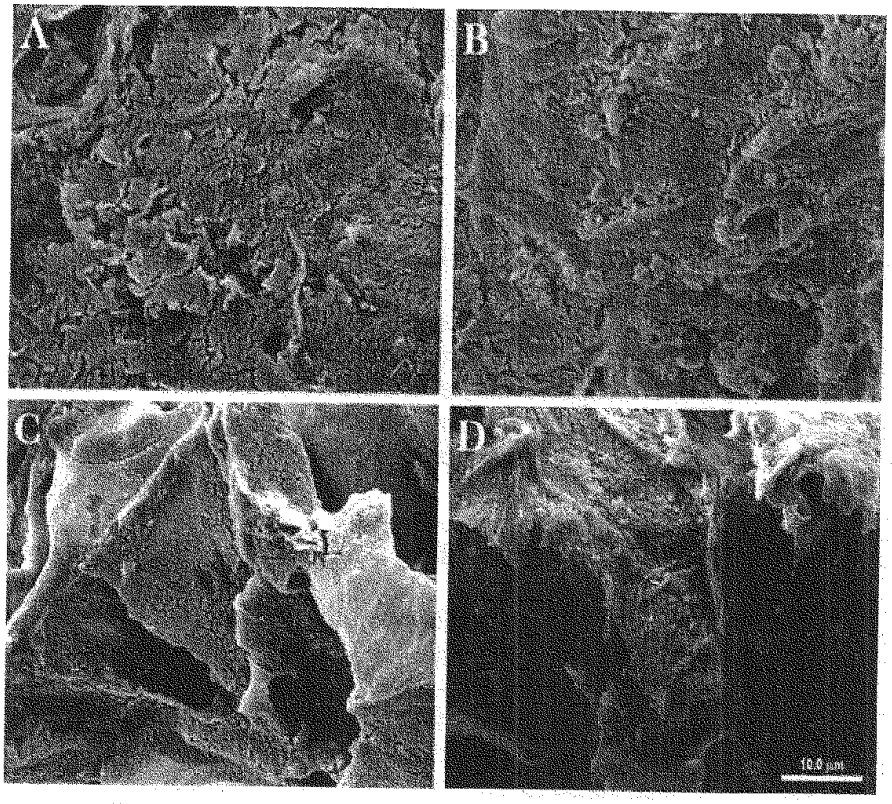

FIG. 5. SCANNING ELECTRON MICROGRAPH (×2500) SHOWING ATTACHMENT AND BIOFILM FORMATION BY *SALMONELLA* CELLS INSIDE THE NETTING OF INOCULATED CANTALOUPES FOLLOWING STORAGE AT 10C

(A) For *Salmonella enterica* sv. Poona RM 2350 after 24 h. (B) For *S. enterica* sv. Poona RM 2350 after 72 h. The cells are arranged within cracks on the netting and bound together by an extracellular matrix. (C) For *S. enterica* sv. Michigan after 24 h. (D) For *S. enterica* sv. Michigan after 72 h.

ture and relative humidity. The aggregate formation by *Salmonella* and *Escherichia coli* O157:H7 on seedlings of *Arabidopsis thaliana* (Cooley *et al.* 2003) and alfalfa sprouts (Charkowski *et al.* 2002) has also been recently presented. Our research agrees with these findings that human pathogens are capable of forming biofilms on plant tissues.

Biofilm formation and attachment of cells to inaccessible sites within the netting (Fig. 1) are likely responsible for the consistent findings among different laboratories that aqueous sanitizers are not effective at removing (or inactivating) bacteria on the surfaces of cantaloupes (Ukuku and Sapers 2001; Annous *et al.* 2004, 2005). Future research must address the fact that patho-

gens adhering to produce exist within biofilms that protect the cells from sanitizers. Sanitizing treatments that are able to penetrate biofilms including surface treatments such as thermal pasteurization (Annous *et al.* 2004) or vapor-phase treatments such as chlorine dioxide gas may be potentially useful in food applications.

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